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(54) Title: TECHNIQUES FOR GROWTH AND DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS

(57) Abstract: This disclosure provides an improved system for culturing human pluripotent stem (pPS) cells in the absence of feeder cells. The role of the feeder cells can be replaced by supporting the culture on an extracellular matrix, and culturing the cells in a conditioned medium. Permanent cell lines are provided that can produce conditioned medium on a commercial scale. Methods have also been discovered to genetically alter pPS cells by introducing the cells with a viral vector or DNA/lipid complex. The system described in this disclosure allows for bulk proliferation of pPS cells for use in studying the biology of pPS cell differentiation, and the production of important products for use in human therapy.

## TECHNIQUES FOR GROWTH AND DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS

### TECHNICAL FIELD

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This invention relates generally to the field of cell biology of embryonic cells. More specifically, it relates to the propagation of human pluripotent stem cells, culture conditions that facilitate propagation, and their use for genetic alteration, producing cDNA libraries, and producing differentiated cells for tissue regeneration.

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### REFERENCE TO RELATED APPLICATIONS

This application claims priority to the following pending U.S. patent applications: USSN 60/175,581, filed January 11, 2000; USSN 60/213,740, filed June 22, 2000; USSN 60/213,739, filed June 22, 2000; USSN 60/216,387, filed July 7, 2000; USSN 60/220,064, filed July 21, 2000; and 09/688,031, filed October 10, 2000.

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For purposes of prosecution in the U.S., the priority applications are hereby incorporated herein by reference in their entirety.

### BACKGROUND

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Recent discoveries have raised expectations that stem cells may be a source of replacement cells and tissues that are damaged in the course of disease, infection, or because of congenital abnormalities. Various types of putative stem cells differentiate when they divide, maturing into cells that can carry out the unique functions of particular tissues, such as the heart, the liver, or the brain. A particularly important discovery has been the development of pluripotent stem cells, which are thought to have the potential to differentiate into almost any cell type.

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Early work on pluripotent stem cells was done in mice (reviewed in Robertson, Meth. Cell Biol. 75:173, 1997; and Pedersen, Reprod. Fertil. Dev. 6:543, 1994). Mouse stem cells can be isolated from both early embryonic cells and germinal tissue. Desirable characteristics of pluripotent stem cells are that they be capable of indefinite proliferation in vitro in an undifferentiated state, retain a normal karyotype, and retain the potential to differentiate to derivatives of all three embryonic germ layers (endoderm, mesoderm, and ectoderm).

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The development of preparations of human pluripotent stem cells has involved overcoming a number of technical difficulties, and is considerably less advanced than work with mouse cells.

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Thomson et al. (U.S. Patent 5,843,780; Proc. Natl. Acad. Sci. USA 92:7844, 1995) were the first to successfully isolate and propagate pluripotent stem cells from primates. They subsequently derived human embryonic stem (hES) cell lines from human blastocysts (Science 282:114, 1998). Gearhart and coworkers derived human embryonic germ (hEG) cell lines from fetal gonadal tissue (Shamblott et al., Proc. Natl. Acad. Sci. USA 95:13726, 1998; and U.S. Patent 6,090,622).

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Both hES and hEG cells are reported to have the long-sought characteristics of pluripotent stem cells: they are capable of long-term proliferation in vitro without differentiating, they have a normal karyotype, and they remain capable of producing a number of different cell types. Because of this, they hold considerable promise for use in human therapy, acting as a reservoir for regeneration of almost any tissue compromised by genetic abnormality, trauma, or a disease condition.

A significant challenge to the use of pluripotent stem cells for therapy is that they are traditionally cultured on a layer of feeder cells to prevent differentiation (U.S. 5,843,780; U.S. 6,090,622). Without feeder cells in the culture environment, hPS cells soon die, or differentiate into a heterogeneous population of committed cells. Leukemia inhibitory factor (LIF) inhibits differentiation of mouse PS cells, but it does not  
5 replace the role of feeder cells in preventing differentiation of human PS cells. Unfortunately, using feeder cells increases production costs, impairs scale-up, and produces mixed cell populations that require the pluripotent stem cells to be separated from feeder cell components.

Another challenge is to control differentiation of stem cells into the particular type of tissue required for treatment of each patient. It is a hypothesis of this invention that better understanding of the differentiation  
10 process will be obtained by observing gene expression during growth and differentiation of pluripotent stem cells.

International Patent Publication WO 99/20741 (Geron Corp.) is entitled *Methods and Materials for the Growth of Primate-Derived Primordial Stem Cells*. A cell culture medium is provided for growing primate-derived primordial stem cells in a substantially undifferentiated state, having a low osmotic pressure and low  
15 endotoxin levels. The basic medium is combined with a serum effective to support the growth of primate-derived primordial stem cells on a substrate of feeder cells or a feeder cell matrix. The medium further includes non-essential amino acids, an anti-oxidant, and growth factors that are either nucleosides or a pyruvate salt.

Sequence-based studies of early human development have focused on libraries produced from fetal  
20 organs and tissues for example, fetal libraries from the I.M.A.G.E. consortium; <http://image.llnl.gov/>). International Patent Publication WO 98/00540 (Incyte) reports partial sequences of stem cell antigens, isolated from cDNA libraries derived from THP-1 cells and bladder tumors.

New technology to facilitate growing and manipulating undifferentiated pluripotent stem cells would be a  
25 major achievement towards realizing the full potential of embryonic cell therapy.

#### SUMMARY OF THE INVENTION

This disclosure provides an improved system for culturing primate pluripotent stem (pPS) cells in the absence of feeder cells. The role of the feeder cells is replaced by supporting the culture on an extracellular  
30 matrix, and culturing the cells in a conditioned medium. Permanent cell lines are provided that can produce conditioned medium on a commercial scale. Methods have also been discovered to genetically alter pPS cells by introducing the cells with a viral vector or DNA/lipid complex. The system described in this disclosure allows for bulk proliferation of pPS cells for use in studying the biology of pPS cell differentiation, and the production of important products for use in human therapy.

35 Described in this disclosure are compositions comprising proliferating pPS cells that are essentially free of feeder cells. The compositions may also comprise a conditioned medium produced by collecting medium from a culture of feeder cells, and a substrate coated with extracellular matrix components. pPS cells may be passaged and expanded in this growth environment in the undifferentiated state.

This disclosure also provides a method for producing a conditioned medium suitable for culturing  
40 primate pluripotent stem (pPS) cells in a growth environment essentially free of feeder cells, comprising conditioning medium by culturing cells in the medium, and harvesting the conditioned medium. The cells used to condition the medium may have one or more desirable features, such as being from a non-malignant source and having a normal karyotype, being capable of extensive culture (such as 60 days or more), have morphological features or cell markers characteristic of fibroblasts, and be immortalized (for example by being

[Biblio](#) [Desc](#) [Claims](#) [Drawing](#)

[Return](#)

59/79 - Claims



CLAIMS

What is claimed as the invention is:

1. A composition comprising proliferating primate pluripotent stem (pPS) cells, which is essentially free of feeder cells.
2. The composition of claim 1, further comprising a conditioned medium produced by collecting medium from a culture of feeder cells.
3. The composition of any preceding claim, further comprising extracellular matrix components (such as Matrigel®, laminin, or collagen).
4. A method for culturing primate pluripotent stem (pPS) cells, comprising culturing pPS cells in a growth environment essentially free of feeder cells but containing conditioned medium produced by collecting medium from a culture of feeder cells.
5. A method for producing a conditioned medium suitable for culturing primate pluripotent stem (pPS) cells in a growth environment essentially free of feeder cells, comprising:
  - a) conditioning medium by culturing cells in the medium, wherein the cells are a euploid cell line that can proliferate in culture for at least 60 days; and
  - b) harvesting the conditioned medium.
6. Conditioned medium to support culturing primate pluripotent stem (pPS) cells in a growth environment essentially free of feeder cells, produced according to the method of claim 5.
7. The composition or method of claims 2-7, wherein the cell line used to produce the conditioned medium has one or more of the following properties:
  - i) it is euploid;
  - ii) it is an immortalized mouse cell line;
  - iii) it is a human cell line;
  - iv) it is a fibroblast cell line; or
  - v) it can proliferate in culture for at least 60 days.
8. A human cell line obtained by differentiating a culture of human embryonic stem (hES) cells into a population of differentiated cells that comprises fibroblast-like cells, and then selecting fibroblast-like cells from the culture; wherein conditioned medium produced by harvesting medium from a culture of the fibroblast-like cells supports growth of pPS cells in a culture environment essentially free of feeder cells.
9. The composition or method of claims 2-8, wherein the cell line used to produce the conditioned medium has been genetically altered to express telomerase reverse transcriptase (TERT) at an elevated level.

10. A method of producing a differentiated cell population, comprising causing or permitting cells of a composition according to claims 1-3, 7, or 9 to differentiate.
11. A method for producing differentiated cells from a donor culture of undifferentiated primate pluripotent stem (pPS) cells, comprising:
  - a) preparing a suspension of cells from the undifferentiated donor culture;
  - b) replating and culturing the suspended cells on a solid surface so that they differentiate without forming embryoid bodies; and
  - c) harvesting differentiated cells from the solid surface.
12. A method for producing differentiated cells from a donor culture of primate pluripotent stem (pPS) cells, comprising:
  - a) providing a culture of primate pluripotent stem (pPS) cells that is essentially free of feeder cells;
  - b) changing the medium in which the cells are cultured; and
  - c) harvesting differentiated cells after culturing for a time in the changed medium.
13. The method of claims 11-12, wherein the donor culture of pPS cells is a culture essentially free of feeder cells, according to any of claims .
14. The method of claims 11-13, having at least one of the following features:
  - i) the solid surface bears a poly-cation (such as poly-lysine or poly-ornithine);
  - ii) differentiation is promoted by withdrawing serum, serum replacement, or a factor that inhibits differentiation from medium in which the cells are cultured after replating; or
  - iii) differentiation is promoted by adding a factor (such as Brain Derived Neurotrophic Factor, BDNF; or Neutrotrophin-3, NT-3) that promotes differentiation in medium in which the cells are cultured after replating.
15. A differentiated cell population produced by the method of any of claims 10-14.
16. A method of screening a compound for cellular toxicity or modulation, comprising contacting a differentiated cell according to claim 15 with the compound, determining any phenotypic or metabolic changes in the cell that result from contact with the compound, and correlating the change with cellular toxicity or modulation.
17. A method for producing a polynucleotide comprising a nucleotide sequence contained in an mRNA that is expressed at a different level in committed or differentiated cells compared with undifferentiated primate pluripotent stem (pPS) cells, the method comprising:
  - a) determining the level of expression of a plurality of mRNAs in committed or differentiated cells, in comparison to the level of expression of the same mRNAs in undifferentiated pPS cells;
  - b) identifying an mRNA expressed at a different level in the committed or differentiated cells, relative to the undifferentiated pPS cells; and
  - c) preparing a polynucleotide comprising a nucleotide sequence of at least 30 consecutive nucleotides contained in the identified mRNA.

18. A method of producing genetically altered primate pluripotent stem (pPS) cells, comprising:
  - a) providing a composition of pPS cells essentially free of feeder cells according to claims 1-3, 7 or 9;
  - b) transferring a polynucleotide into pPS cells in the composition; and then optionally
  - c) preferentially selecting cells that have been genetically altered with the polynucleotide.
19. A method of producing genetically altered primate pluripotent stem (pPS) cells, comprising:
  - a) providing a composition of pPS cells on a layer of feeder cells that are drug-resistant;
  - b) transferring a polynucleotide into pPS cells in the composition; and
  - c) selecting genetically altered cells in the composition using the drug to which the feeder cells are resistant.
20. The method of claims 18-19, wherein the polynucleotide comprises a protein encoding region operably linked to a promoter that promotes transcription of the encoding region in an undifferentiated pPS cell.
21. A population of primate pluripotent stem (pPS) cells, in which at least 25% of the undifferentiated pPS cells have been stably transfected with a polynucleotide, or are the progeny of such cells that have inherited the polynucleotide.
22. A population of genetically altered differentiated cells, obtained by differentiating the cells of claim 21.
23. A method of producing an mRNA preparation or a cDNA library from primate pluripotent stem (pPS) cells before or after differentiation, comprising providing a culture of undifferentiated pPS cells essentially free of feeder cells, optionally permitting the pPS cells to differentiate, and isolating mRNA from the undifferentiated or differentiated cells.
24. The method of claim 23, comprising isolating mRNA from pPS cells in a culture essentially free of feeder cells, and recombining cDNA copies of the mRNA into a cloning vector, wherein the cDNA copies are operatively linked to a transcriptional regulatory control element (such as the PGK promoter) that promotes transcription of the cDNA in undifferentiated pPS cells.
25. The method of claims 23-24, which is a method for producing a cDNA subtraction library enriched for transcripts differently expressed in a first cell population compared with a second cell population, comprising incubating together preparations of mRNA (or cDNA copies thereof) obtained from the first and second cell populations under conditions that permit polynucleotides present in both preparations to cross-hybridize; and then recombining polynucleotides that have not cross-hybridized into a cloning vector.
26. A cDNA library produced according to the method of any of claims 23-25.
27. A cDNA library of at least 1,000 genes expressed at the mRNA level in either undifferentiated pPS cells, or cells differentiated from pPS cells, wherein the library is essentially free of cDNA of other vertebrates.

28. The cDNA library of claims 26-27, wherein at least 30% of cDNA segments in the library comprise the entire encoding region of the corresponding mRNA.
29. A method for producing a polynucleotide containing a sequence of an mRNA expressed in undifferentiated or differentiated pPS cells, comprising determining nucleotide sequence from an mRNA or cDNA obtained according to claims 23-25, and manufacturing a polynucleotide containing the determined sequence.
30. A method for producing an amino acid containing a sequence of a polypeptide expressed in undifferentiated or differentiated pPS cells, comprising determining amino acid sequence from a protein encoding region of an mRNA or cDNA obtained according to claims 23-25, and manufacturing a protein containing the determined sequence.
31. A method for producing an antibody specific for a polypeptide expressed in undifferentiated or differentiated pPS cells, comprising determining amino acid sequence from a protein encoding region of an mRNA or cDNA obtained according to claims 23-25, and immunizing an animal or contacting an immunocompetent cell or particle with a protein containing the determined sequence.
32. The method or composition of any preceding claim, wherein the pPS cells are human embryonic stem (hES) cells.



18. A method of producing genetically altered primate pluripotent stem (pPS) cells, comprising:
  - a) providing a composition of pPS cells essentially free of feeder cells according to claims 1-3, 7 or 9;
  - b) transferring a polynucleotide into pPS cells in the composition; and then optionally
  - c) preferentially selecting cells that have been genetically altered with the polynucleotide.
19. A method of producing genetically altered primate pluripotent stem (pPS) cells, comprising:
  - a) providing a composition of pPS cells on a layer of feeder cells that are drug-resistant;
  - b) transferring a polynucleotide into pPS cells in the composition; and
  - c) selecting genetically altered cells in the composition using the drug to which the feeder cells are resistant.
20. The method of claims 18-19, wherein the polynucleotide comprises a protein encoding region operably linked to a promoter that promotes transcription of the encoding region in an undifferentiated pPS cell.
21. A population of primate pluripotent stem (pPS) cells, in which at least 25% of the undifferentiated pPS cells have been stably transfected with a polynucleotide, or are the progeny of such cells that have inherited the polynucleotide.
22. A population of genetically altered differentiated cells, obtained by differentiating the cells of claim 21.
23. A method of producing an mRNA preparation or a cDNA library from primate pluripotent stem (pPS) cells before or after differentiation, comprising providing a culture of undifferentiated pPS cells essentially free of feeder cells, optionally permitting the pPS cells to differentiate, and isolating mRNA from the undifferentiated or differentiated cells.
24. The method of claim 23, comprising isolating mRNA from pPS cells in a culture essentially free of feeder cells, and recombining cDNA copies of the mRNA into a cloning vector, wherein the cDNA copies are operatively linked to a transcriptional regulatory control element (such as the PGK promoter) that promotes transcription of the cDNA in undifferentiated pPS cells.
25. The method of claims 23-24, which is a method for producing a cDNA subtraction library enriched for transcripts differently expressed in a first cell population compared with a second cell population, comprising incubating together preparations of mRNA (or cDNA copies thereof) obtained from the first and second cell populations under conditions that permit polynucleotides present in both preparations to cross-hybridize; and then recombining polynucleotides that have not cross-hybridized into a cloning vector.
26. A cDNA library produced according to the method of any of claims 23-25.
27. A cDNA library of at least 1,000 genes expressed at the mRNA level in either undifferentiated pPS cells, or cells differentiated from pPS cells, wherein the library is essentially free of cDNA of other vertebrates.